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Processing *O*-glycan core 1, Galβ1-3GalNAcα-R. Specificities of core 2, UDP-GlcNAc: Galβ1-3GalNAc-R(GlcNAc to GalNAc) β6-*N*-acetylglucosaminyltransferase and CMP-sialic acid:Galβ1-3GalNAc-R α3-sialyltransferase

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To elucidate control mechanisms of *O*-glycan biosynthesis in leukemia and to develop biosynthetic inhibitors we have characterized core 2 UDP-GlcNAc:Galβ1-3GalNAc-R(GlcNAc to GalNAc) β6-*N*-acetylglucosaminyltransferase (EC 2.4.1.102; core 2 β6-GlcNAc-T) and CMP-sialic acid: Galβ1-3GalNAc-R α3-sialyltransferase (EC 2.4.99.4; α3-SA-T), two enzymes that are significantly increased in patients with chronic myelogenous leukemia (CML) and acute myeloid leukemia (AML). We observed distinct tissue-specific kinetic differences for the core 2 β6-GlcNAc-T activity; core 2 β6-GlcNAc-T from mucin secreting tissue (named core 2 β6-GlcNAc-T M) is accompanied by activities that synthesize core 4 [GlcNAcβ1-6(GlcNAcβ1-3)GalNAc-R] and blood group I [GlcNAcβ1-6(GlcNAcβ1-3)Galβ-R] branches; core 2 β6-GlcNAc-T in leukemic cells (named core 2 β-GlcNAc-T L) is not accompanied by these two activities and has a more restricted specificity. Core 2 β6-GlcNAc-T M and L both have an absolute requirement for the 4- and 6-hydroxyls of *N*-acetylgalactosamine and the 6-hydroxyl of galactose of the Galβ1-3GalNAcα-benzyl substrate but the recognition of other substituents of the sugar rings varies, depending on the tissue. α3-sialytransferase from human placenta and from AML cells also showed distinct specificity differences, although the enzymes from both tissues have an absolute requirement for the 3-hydroxyl of the galactose residue of Galβ1-3GalNAcα-Bn. Galβ1-3(6-deoxy)GalNAcα-Bn and 3-deoxy-Galβ1-3GalNAcα-Bn competitively inhibited core 2 β6-GlcNAc-T and α3-sialyltransferase activities, respectively.

Keywords: glycosyltransferase; *O*-glycan; β6-*N*-acetylglucosaminyltransferase; α3-sialyltransferase; specificity; leukemia.

Abbreviations: AFGP, antifreeze glycoprotein; AML, acute myeloid leukemia; Bn, benzyl; CML, chronic myelogenous leukemia; Fuc, L-fucose; Gal, G, D-galactose; GalNAc, GA, *N*-acetyl-D-galactosamine; GlcNAc, Gn, *N*-acetyl-D-glucosamine; HC, human colonic homogenate; HO, hen oviduct microsomes; HPLC, high performance liquid chromatography; mco, 8-methoxycarbonyl-octyl; Me, methyl; MES, 2-(*N*-morpholino)ethanesulfonate; MK, mouse kidney homogenate; onp, *o*-nitrophenyl; PG, pig gastric mucosal microsomes; pnp, *p*-nitrophenyl; RC rat colonic mucosal microsomes; SA, sialic acid; T, transferase.

Enzymes: UDP-GlcNAc:Galβ1-3GalNAc-R (GlcNAc to GalNAc) β6-*N*-acetylglucosaminyltransferase, *O*-glycan core 2 β6-GlcNAc-transferase, EC 2.4.1.102; CMP-sialic acid: Galβ1-3GalNAc-R α3-sialyltransferase, *O*-glycan α3-sialic acid-transferase, EC 2.4.99.4.

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The biological role of protein-linked complex carbohydrates is potentially important to development, cell growth, differentiation, cancer and metastasis. Leukocytes express highly sialylated glycoproteins on their cell surfaces that are rich in GalNAc-Ser/Thr-linked oligosaccharides (*O*-glycans). These *O*-glycans change during differentiation and in leukemias [1–5]. Processing of *O*-glycan core 1, Galβ1-3GalNAcα-R (Fig. 1) may proceed through elongation, sialylation, sulfation, fucosylation, branching and addition of blood group epitopes [6]. The direction of pathways in *O*-glycan biosynthesis is regulated by the specificities of the glycosyltransferases and by their relative activities, and other control factors.

In previous studies we noted a reduction in α2-fucosyltransferase activity in leukemia cells [7] and the absence of the elongation β3-GlcNAc-transferase that elongates cores 1 and 2 by addition of a β1-3 linked GlcNAc residue (paths a and g, respectively, Fig. 1) [8]. However, two activities were found significantly increased in granulocytes and blast cells from patients with chronic myelogenous leukemia (CML) and in blast cells from patients with acute myeloid leukemia (AML): UDP-GlcNAc:Galβ1-3GalNAc-R (GlcNAc to GalNAc) β6-GlcNAc-transferase (EC 2.4.1.102; core 2 β6-GlcNAc-T), the enzyme synthesizing *O*-glycan core 2 (path b, Fig. 1) [8], and CMP-sialic acid:Galβ1-3GalNAc-R α3-sialyltransferase (EC 2.4.99.4; α3-SA-T) (path c, Fig. 1) [9–11]. Both enzymes may contribute to the hypersialylation and aberrant growth and functions of leukemic cells.

Core 2 β6-GlcNAc-T is present in most tissues [12–17]. The enzyme has recently been purified from bovine trachea [18], and its gene has been cloned from HL60 cells [19]. Core 2 β6-GlcNAc-T activity undergoes variations during the differentiation of human colonic adenocarcinoma CaCo-2 cells [20] and is increased in AML and CML [8] and in acute and chronic lymphoid leukemias (ALL and CLL) [3]. The activity increases upon activation of human lymphocytes [21] and is elevated in lymphocytes from patients with the Wiscott–Aldrich syndrome [22, 23]. Certain metastatic murine tumour cells are associated with high core 2 β6-GlcNAc-T activity [24]. Substrate specificity studies on core 2 β6-GlcNAc-T from mucin secreting tissues of pig and cow [16, 18, 25] suggested that a single enzyme catalyses the addition of a β6-GlcNAc branch to form both *O*-glycan cores 2 and 4 and to synthesize the partial blood group I epitope (GlcNAcβ1-6[GlcNAcβ1-3]Galβ) on *O*-glycans, N-glycans and glycolipids. However, only the core 2 synthesizing activity is present in certain leukemic cells and in human colonic adenocarcinoma CaCo-2 cells; the activities synthesizing core 4 or the blood group I branch are virtually absent [8, 20].

The α3-SA-T that sialylates *O*-glycan core 1 has been characterized and purified from porcine submaxillary glands [26, 27] and from human placenta [28]. The enzyme acts on Galβ1-3GalNAc-R structures on glycoproteins and ganglioside GM_1 and on low molecular weight acceptors with Galβ1-3 GalNAcα/β- termini [28, 29]. Other terminal structures such as Galβ1-3GlcNAc- are much less effective

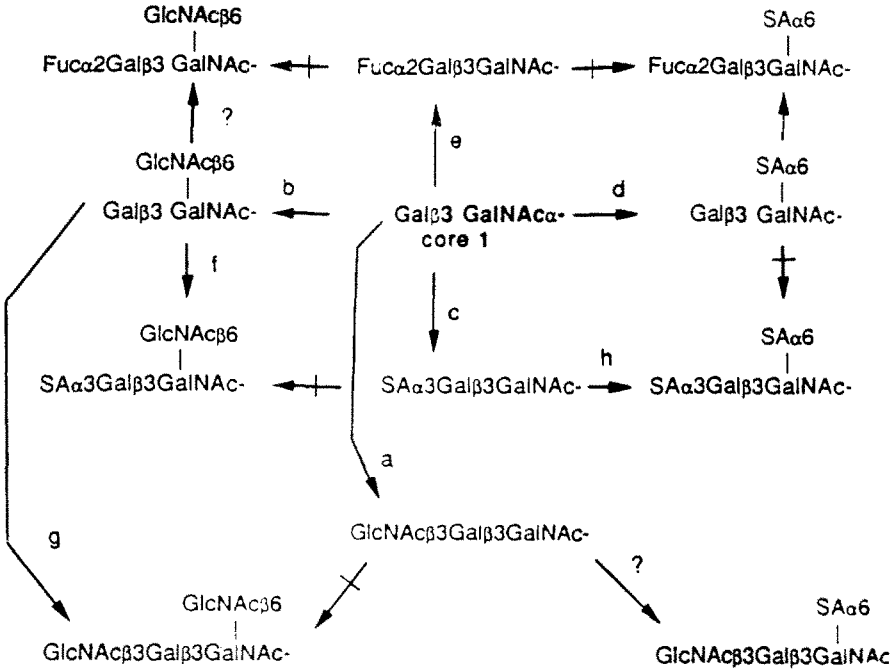

Figure 1. Biosynthetic processing of *O*-glycan core 1, Galβ1-3GalNAc-. Paths a and g, elongation β3-GlcNAc-transferase; path b, core 2 β6-GlcNAc-transferase; paths c and f, α3-SA-transferase; path d, α6-SA-transferase I; path e, α2-Fuc-transferase; path h, α6-SA-transferase II. Bars across the arrows indicate that these pathways are blocked.

acceptors. The enzyme appears to be increased in cells from leukemia patients [9,10] and in leukemic cell lines during differentiation [11]. The gene-coding for the enzyme has been cloned from porcine submaxillary glands [30].

In this paper, we report substrate specificity and kinetic studies of core 2 β6-GlcNAc-T and α3-SA-T from several tissues and show that the kinetic properties of both transferases vary with the tissue. We also describe competitive inhibitors for core 2 β6-GlcNAc-T and α3-SA-T in leukemic cells. Since hypersialylation of leukemic cells may be responsible for the aberrant functions of these cells, such inhibitors are potential therapeutic agents for leukemia.

Materials and methods

Materials

Acetonitrile was obtained from Caledon Laboratories and Fisher Scientific Co. AG 1-X8 (100–200 mesh, Cl^- form) was purchased from Bio-Rad. Triton X-100, UDP-Gal, GlcNAc, GlcNAcβ1-3Galβ-Me, Galβ1-3GalNAcα-phenyl and Galβ1-3GalNAcα-Bn were from Sigma. All other commercial reagents were of the finest grade. UDP-*N*-[1-^{14}C]acetylglucosamine was prepared as described previously [15] and diluted with non-radioactive UDP-GlcNAc from Sigma. CMP-[^{14}C]SA was purchased from Amersham International and diluted with CMP-SA from Sigma. Galβ1-3GalNAcα-*p*-nitrophenyl was purchased from Toronto Research Chemicals, Toronto. Antifreeze glycoprotein was donated by Dr Choy Hew, University of Toronto. Bovine testicular β-galactosidase was a gift from Dr W. G. Jourdian, Ann Arbor, MI. Purified α3-SA-T from human placenta was purchased from Genzyme.

Preparation of oligosaccharides

The following oligosaccharides were synthesized chemically: SAcα2-3Galβ1-3GalNAc [31]; 3-deoxy-Galβ1-3GalNAcα-Bn; 4-deoxy-Galβ1-3GalNAcα-Bn; 6-deoxy-Galβ1-3GalNAcα-Bn; Galβ1-3(4-deoxy)GalNAcα-Bn; Galβ1-3(6-deoxy) GalNAcα-Bn, Galβ1-3(2-deoxy)Galα-Bn [32]; Galβ1-3GalNAcα-mco [33]; Galβ1-3GalNAcαSer [34]; Ala-Pro-(Galβ1-3GalNAcα)Ser-Ser-Ser; Ala-Pro-(Galβ1-3GalNAcα)Thr-Ser-Ser [35]; Galβ1-3GlcNAcβ-Bn [36]; GlcNAcβ1-6(Galβ1-3)GalNAcα-Bn, GlcNAcβ1-6-(GlcNAcβ1-6Galβ1-3)GalNAcα-Bn [37]; GlcNAcβ1-6(D-Fucβ1-3)GalNAcα-Bn [38]; Galβ1-3GalNAcα-*o*-nitrophenyl [39]; Galβ1-3GalNAc [40]; and GlcNAcβ1-3-GalNAcα-Bn [41]. Galβ1-3(6-*O*-(4,4-azo)pentyl) GalNAcα-Bn was synthesized from 6-*O*-(4,4-azo)pentyl-GalNAcα-Bn [42] by a scaled-up procedure using partially purified rat liver core 1 β3-Gal-transferase, as described previously [43]. The formation of the water-soluble product was followed by the disappearance of water-insoluble substrate and by thin layer chromatography in acetonitrile:water = 7:1 by vol. The product was purified by successive chromatography on columns of AG1-X8 (100–200 mesh) and Bio-Gel P-2 (equilibrated in water) followed by adsorption and elution from a C_{18} Sep-Pak cartridge. Purified product was obtained in 62% yield. The galactose content was analysed after hydrolysis by high performance anion exchange chromatography with pulsed amperometric detection (Dionex system). The product was characterized by 500 MHz proton NMR spectroscopy (see Table 4). The addition of galactose to 6-*O*-(4,4-azo)pentyl-GalNAcα-Bn is shown by the H-1 and H-4 signals of galactose. The attachment of the Gal residue at the 3-position of GalNAc has a significant effect on the signals of H-2, H-3, H-4 and H-5 of GalNAc.

HPLC

HPLC separations were performed with an LKB or a Waters system as described previously [8, 20, 44, 45]. Columns were protected with a guard column filled with C_{18}-bound silica. A 10 μ C_{18} column (8 mm × 100 mm) was used with acetonitrile:water as the mobile phase at a flow rate of 1 ml min^{-1}. Pressures varied between 40 bar and 100 bar according to the type of column, the age of the column, the flow rate and the mobile phase concentration. Elutions were monitored by measuring the absorbance at 195 nm and counting the radioactivity of collected fractions after the addition of 10 ml of scintillation fluid (Readysolv from Beckman) with an LKB model 1209 scintillation counter.

High voltage paper electrophoresis

Samples were applied on Whatman paper. High voltage electrophoresis (HVE) was run in 1% sodium tetraborate buffer at 150 mA and 1500 V (~4 mA cm^{-1}) for 65–90 min. The paper was dried and cut into 2 cm strips. Radioactivity was counted in 15 ml scintillation fluid (Readysolv by Beckman).

Methylation analysis

Methylation analysis was carried out as described [45] following the method of Ciucanu and Kerek [46].

Mass spectrometry

FAB mass spectra were recorded with a VG Analytical ZAB-SE double focusing mass spectrometer in the Toronto Carbohydrate Research Center, and electron impact mass spectrometry was carried out at the University of Toronto as described [45].

Proton NMR spectroscopy

Samples were prepared in ^2H_2O and analysed as described previously [45] 1- and 2-Dimensional (COSY) proton NMR spectra were recorded with a 500 MHz Bruker spectrometer in the Toronto Carbohydrate Research Center with acetone as the internal standard set at 2.225 ppm.

Protein determinaion

Protein was determined by Bio-Rad method [47] with bovine serum albumin as the standard.

β-Galactosidase digestion

Enzyme product was subjected to digestion by bovine testicular β-galactosidase as follows. Each 100 µl contained 25 µl citrate-phosphate (McIlvaine) buffer, pH 4.3, 0.1 µg bovine serum albumin, 3–5 nmol (2000–3200 disintegrations per minute, dpm) of enzyme product and 4 µl containing 6 munits (1 munits = 1 nmol min^{-1}) bovine testicular β-galactosidase for 24 h at 37 °C. Control incubations lacked the enzyme. Reactions were stopped by heating for 10 min at 80 °C and lyophilizing. Product was analysed by HPLC and compared with the control, using a C_{18} column with acetonitrile:water (8:92 by vol) as the mobile phase at 1 ml min^{-1}. The activity of the β-galactosidase preparation was tested in a mixture containing 5 mM Galβ-*p*-nitrophenyl, 25 µl McIlvaine buffer, pH 4.3, 0.1 µg bovine serum albumin and 1 µl enzyme solution in a total volume of 100 µl, incubated for 30 min at 37 °C, followed by the addition of 1 ml 0.25 M glycine buffer, pH 10, and absorbance measurements at 400 nm [48].

Enzyme preparations

AML blast cells were isolated from patients with acute myeloblastic leukemia, and CML granulocytes were isolated from patients with chronic myelogenous leukemia as described previously [8]. Human and rat colonic homogenates and rat colonic and pig gastric mucosal microsomes were prepared as described [15]. The preparation of hen oviduct microsomes was described previously [44]. Mouse kidney homogenates were prepared from adult mouse kidneys by hand homogenization in 0.25 M sucrose.

Enzyme assays for core 2 β6-GlcNAc-T

Standard assay mixtures for the core 2 β6-GlcNAc-transferase contained the following ingredients in a total volume of 40 µl: 0.125 M GlcNAc, 0.125 M MES buffer, pH 7, 0.25% Triton X-100, 2.5 mM ATP or 10 mM AMP, 5 mM γ-galactonolactone, 2 mM Galβ1-3GalNAcα-Bn, 0.8–4.5 mM UDP-[^{14}C]GlcNAc (400–3000 dpm $nmol^{-1}$) and 10–20 µl cell homogenate or microsomes (0.1–0.38 mg protein). Mixtures were incubated for 1 h at 37 °C. Reactions were stopped by the addition of 0.4 ml 20 mM sodium tetraborate/1 mM EDTA. Mixtures were passed through Pasteur pipettes filled with AG 1-X8, Cl^- form, 100–200 mesh, followed by washing with 2.6 ml water. Eluates were either counted directly in 17 ml scintillation fluid, or were lyophilized, taken up in 200 µl water, and stored frozen. Aliquots of 100 µl were subsequently analysed by HPLC. Incubations using oligosaccharide substrates with free reducing ends were stopped with 10 µl 25 mM EDTA/2% tetraborate and analysed by high voltage paper electrophoresis followed by descending paper chromatography overnight with 80% ethanol.

Substrate specificity studies were carried out in a total volume of 40 µl containing 0.125 M GlcNAc, 0.125 M MES buffer, pH 7, 0.25% Triton X-100, 5 mM AMP, 5 mM γ-galactonolactone, 4.6 mM UDP-[^{14}C]GlcNAc (650 dpm $nmol^{-1}$), and 20 µl AML blast cell homogenate (0.22 mg protein) and substrate at four to six different concentrations. Mixtures were incubated for 1 h at 37 °C. Radioactive product was determined as described above without HPLC. Kinetic parameters were determined by linear reciprocal Lineweaver–Burk plots.

Inhibition studies were carried out at four or five different inhibitor concentrations (0–5 mM). In studies on the diazirine-containing inhibitor, the enzyme was preincubated in the incubation mixture with the diazirine for 10 min followed by irradiation with a Rayonet RPR 100 photochemical reactor with 16 lamps (RPR 3500 A) before the addition of substrate.

Enzyme assays for α3-SA-T

Purified human placental α3-SA-T. The standard incubation mixture contained in a total volume of 25 µl: 2 µmol Tris-HCl, pH 7.07, 0.4% Triton X-100, 0.5–0.9 mM CMP-[^{14}C]SA (2880–4400 dpm $nmol^{-1}$), 0.04 µg bovine serum albumin, 3.2 mM substrate and 1 µl enzyme. Mixtures were incubated for 1 h at 37 °C; 10 µl 25 mM EDTA/2% borate were added and mixtures were frozen. Samples were applied on Whatman 3 MM filter paper for high voltage electrophoresis.

AML α3-SA-T. The standard incubation mixture contained in a total volume of 40 µl: 4 µmol Tris-HCl, pH 7.5, 0.25% Triton X-100, 0.93 mM CMP-[^{14}C]SA (2880–4400 dpm $nmol^{-1}$), 2 mM substrate and 20 µl enzyme (0.1 to 0.2 mg protein). Mixtures were incubated for 1 h at 37 °C; 10 µl 25 mM EDTA/2% borate were added and mixtures were frozen. Samples were applied on Whatman 3 MM filter paper for high voltage electrophoresis.

Isolation of large scale core 2 β6-GlcNAc-T products

1. Core 2 β6-GlcNAc-T product using 3-deoxy-Galβ1-3GalNAcα-Bn substrate. To prepare enzyme product using 3-deoxy-Galβ1-3GalNAcα-Bn substrate for structural analysis, the incubation was scaled up as follows. The incubation mixture contained in a total volume of 1 ml: 2 mM 3-deoxy-Galβ1-3GalNAcα-Bn, 0.2 M GlcNAc, 0.1 M MES buffer, pH 7, 8 mM ATP, 0.2% Triton X-100, 4-mM γ-galactonolactone, 2.5 mM UDP-GlcNAc (474 dpm $nmol^{-1}$) and 400 µl rat colonic homogenate (6.4 mg protein). The mixture was incubated at 37 °C for 4 h; 4 ml 20 mM borate/1 mM EDTA were added, and the mixture was applied to a column (1.5 cm × 10 cm) filled with AG 1-X8 and eluted with 60 ml water. The eluant was lyophilized and product was purified on Bio-Gel P-4 (1.6 cm × 90 cm), equilibrated in water, followed by HPLC using a C_{18}

column, run at 1 ml min^{-1} with acetonitrile:water (8:92 by vol) as the mobile phase.

2. Core 2 β6-GlcNAc-T product using 4-deoxy-Galβ1-3GalNAcα-Bn substrate. To prepare enzyme product using 4-deoxy-Galβ1-3GalNAcα-Bn substrate for structural analysis, the incubation was scaled up as follows. The incubation mixture contained in a total volume of 1 ml: 6 mM 4-deoxy-Galβ1-3GalNAcα-Bn, 0.25 M GlcNAc, 0.25 M MES buffer, pH 7, 20 mM ATP, 0.5% Triton X-100, 10 mM γ-galactonolactone, 2.5 mM UDP-GlcNAc (637 dpm $nmol^{-1}$) and 500 µl rat colonic homogenate (8 mg protein). The mixture was incubated at 37 °C for 3 h; 4 ml 20 mM borate/1 mM EDTA were added, and the mixture was applied to a column (1.5 cm × 10 cm) filled with AG 1-X8 and eluted with 60 ml water. The eluant was lyophilized and product was purified on Bio-Gel P-4 (1.6 cm × 90 cm), equilibrated in water, followed by HPLC using a C_{18} column, run at 1 ml min^{-1} with acetonitrile:water (8:92 by vol) as the mobile phase.

3. Core 2 β6-GlcNAc-T product using Galβ1-3(2-deoxy)-Galα-Bn substrate. To prepare enzyme product using Galβ1-3(2-deoxy)Galα-Bn substrate for structural analysis, the incubation was scaled up as follows. The incubation mixture contained in a total volume of 1 ml: 2 mM Galβ1-3(2-deoxy)Galα-Bn, 0.2 M GlcNAc, 0.1 M MES buffer, pH 7, 8 mM ATP, 0.2% Triton X-100, 4 mM γ-galactonolactone, 2.4 mM UDP-GlcNAc (465 dpm $nmol^{-1}$) and 400 µl hen oviduct microsomes (21.2 mg protein). The mixture was incubated at 37 °C for 4 h; 4 ml 20 mM borate/1 mM EDTA were added, and the mixture was applied to a column (1.5 cm × 10 cm) filled with AG 1-X8 and eluted with 60 ml water. The eluant was lyophilized and product was purified on Bio-Gel P-4 (1.6 cm × 90 cm), equilibrated in water, followed by HPLC using a C_{18} column, run at 1 ml min^{-1} with acetonitrile:water (8:92 by vol) as the mobile phase.

Results

Substrate specificity and tissue distribution of core 2 β6-GlcNAc-transferase

The core 2 β6-GlcNAc-T from AML cells acts on substrates with the general structure Galβ1-3GalNAc-R (Table 1) where R may be H, a hydrophobic group, Ser or Thr, or a peptide. We found significant kinetic differences between these substrates. Although the enzyme acts well on Galβ1-3GalNAc with a free reducing end, the length and structure of the aglycon influences the activity considerably. The addition of α3 linked sialic acid to galactose or substitution of the 6-hydroxyl of GalNAc completely inhibited the formation of product (Table 1).

We determined the tissue distribution of core 2 β6-GlcNAc-T activity towards Galβ1-3GalNAcα-Bn substrate and its monodeoxy derivatives (Tables 2 and 3). 3-Deoxy-Galβ1-3GalNAcα-Bn showed greatly reduced activity relative to Galβ1-3GalNAcα-Bn with enzyme from pig gastric microsomes but retained appreciable activity at different levels with the other tissues tested. 4-Deoxy-Galβ1-3GalNAcα-Bn showed relatively low activity in all tissues except for rat colon. Galβ1-3(2-deoxy)Galα-Bn was fully active with hen oviduct microsomes but appreciably reduced with other tissues. 6-Deoxy-Galβ1-3GalNAcα-Bn, Galβ1-3(6-deoxy)GalNAcα-Bn, Galβ1-3(4-deoxy)GalNAcα-Bn and Galβ1-3[6-*O*-(4,4-azo)pentyl]GalNAcα-Bn were ineffective substrates in all tissues tested (Tables 1–3).

Identification of large scale enzyme products using 3-deoxy-Galβ1-3GalNAcα-Bn, 4-deoxy-Galβ1-3GalNAcα-Bn and Galβ1-3(2-deoxy)Galα-Bn substrates.

(1) Large scale enzyme product A using 3-deoxy-Galβ1-3GalNAcα-Bn substrate was separated by gel filtration using Bio-Gel P-4. Product A eluted from the P-4 column

Table 1. Specificity of core 2 β6-GlcNAc-transferase from acute myeloid leukemic blast cells.[a]

Substrate	K_M (mM)	V_{max} (nmol h^{-1} mg^{-1})	V_{max}/K_M
Galβ3GalNAcα-Bn	0.8	25	31
Galβ3GalNAcα-pnp	0.6	27	45
Galβ3GalNAcα-onp	2.0	50	25
Galβ3GalNAcα-phenyl	10	50	5
Galβ3GalNAcα-mco	0.4	44	110
Galβ3GalNAcα-Ser	0.3	8	27
Ala-Pro-(Galβ3GalNAcα)Ser-Ser-Ser	0.8	11	14
Ala-Pro-(Galβ3GalNAcα)Thr-Ser-Ser	1.4	29	21
Galβ3GalNAcα-AFGP	1.3	33	25
Galβ3GalNAc	3.8	80	21
SAα3Galβ3GalNAc	nd[b]	nd	
Galβ3[6-*O*-(4,4-azo)pentyl]GalNAcα-Bn	nd	nd	

[a] Assays were carried out as described in the Materials and methods section.
[b] nd, not determined, since the activity was <0.1 nmol h^{-1} mg^{-1}.

Table 2. Kinetic parameters of core 2 β6-GlcNAc-transferase from different tissues.[a]

Substrate	*Tissue*[b]	K_M (mM)	V_{max} (nmol h^{-1} mg^{-1})	V_{max}/K_M
Galβ3GalNAcα-Bn	AML	1.7	50	29
	PG	1.1	217	193
	HC	1.4	104	74
	RC	1.2	303	253
	RCH	1.3	172	132
	MK	2.0	500	250
	HO	1.1	26	24
3-deoxy-Galβ3GalNAcα-Bn	AML	3.3	74	22
	PG	5.5	40	7.3
	HC	4.5	45	8
	RC	4.3	526	122
	MK	2.0	375	188
4-deoxy-Galβ3GalNAcα-Bn	AML	6.7	5	0.75
	PG	5	100	20
	HC	10	25	2.5
	RC	4.2	667	159
	MK	3.1	263	85
Galβ3(2-deoxy)Galα-Bn	RCH	3.3	50	15
	HO	1.9	39	21

[a] Assays were carried out as described in the Materials and methods section.
[b] AML, acute myeloid leukemia cell homogenate; PG, pig gastric microsomes; HC, human colonic homogenate; RC, rat colonic microsomes; RCH, rat colon homogenate; MK, mouse kidney homogenate; HO, hen oviduct microsomes.

Table 3. Competition experiments using Galβ1-3GalNAcα-Bn substrate and substrate analogues in the assay for core 2 β6-GlcNAc-transferase.

Substrate	*Analogue*	*Enzyme activities* (nmol h^{-1} mg^{-1}[a])					
		AML[b]	*PG*[c]	*HC*[c]	*RC*[c]	*MK*[d]	*HO*
Galβ3GalNAcα-Bn	–	50	112	61	207	75	–
–	3-deoxy-Galβ3GalNAcα-Bn	46	14	24	142	22	–
Galβ3GalNAcα-Bn	3-deoxy-Galβ3GalNAcα-Bn	54	120	65	302	68	–
–	4-deoxy-Galβ3GalNAcα-Bn	3.0	10.1	6.7	187	8.6	–
Galβ3GalNAcα-Bn	4-deoxy-Galβ3GalNAcα-Bn	60	126	62	380	77	–
–	6-deoxy-Galβ3GalNAcα-Bn	0.3	0	0	6.1	1.1	–
Galβ3GalNAcα-Bn	6-deoxy-Galβ3GalNAcα-Bn	50	126	64	210	78	–
–	Galβ3(4-deoxy)GalNAcα-Bn	1.8	1.5	0	15	0	–
Galβ3GalNAcα-Bn	Galβ3(4-deoxy)GalNAcα-Bn	46	126	76	233	82	–
–	Galβ3(6-deoxy)GalNAcα-Bn	1.4	0	0	0	0.2	–
Galβ3GalNAcα-Bn	Galβ3(6-deoxy)GalNAcα-Bn	50	113	67	223	69	–
Galβ3GalNAcα-Bn[e]	–	32	129	27	54	176	34
–	Galβ3(2-deoxy)Galα-Bn	0.7	29	1.1	16	33	34
Galβ3GalNAcα-Bn	Galβ3(2-deoxy)Galα-Bn	32	120	31	56	160	40

[a] Enzyme assays were carried out as described in the Materials and methods section. Values represent the average of at least two determinations. Assays with AML and MK, and all experiments using Galβ1-3(2-deoxy)GalNAcα-Bn were carried out by HPLC as follows. Compounds were separated by HPLC on a C_{18} column with acetonitrile:water ratios ranging from 10:90 to 13:87 (by vol) at a flow rate of 1 ml min^{-1}. Products always elute earlier (17–22 min) than their respective substrates (24–54 min).
[b] Substrate and analogue concentrations in the assays were 2 mM.
[c] Substrate and analogue concentrations in the assays were 1.5 mM.
[d] Substrate and analogue concentrations in the assays were 1 mM.
[e] Separate experiment with different enzyme preparations. The substrate and analogue concentrations were 2 mM for AML, PG, HC, MK and HO, and 1 mM for RC. AML, PG, HC, RC, MK, HO as described in Table 2.

as well as on reversed phase HPLC well separated from the substrate. Product A did not change its elution pattern on HPLC after β-galactosidase digestion, suggesting that A is resistant to β-galactosidase. The NMR spectrum of product A (Table 4) compared with the substrate indicate the addition of β1-6-linked GlcNAc residue with a resonance at 1.964 ppm due to the *N*-acetyl group and a doublet due to H-1 at 4.544 ppm with a coupling constant $J_{1,2}$ of 8.7 Hz. The product A spectrum showed an upfield shift of the upfield resonance of the benzyl CH_2 proton from 4.574 to 4.505 ppm, typical for the attachment of β6-linked GlcNAc to GalNAc [16]. For comparison, the chemical shift values for Galβ1-3GalNAcα-Bn and GlcNAcβ1-6-(Galβ1-3)GalNAcα-Bn are included in Table 4. The masses determined by FAB mass spectrometry were *m/z* 458 $[M + H]^+$ and 480 $[M + Na]^+$ for the substrate 3-deoxy-Galβ1-3GalNAcα-Bn and *m/z* 661 $[M + H]^+$ and 683 $[M + Na]^+$ for product A. Based on the mass spectrometric and NMR data, the structure of enzyme product A is consistent with [^{14}C]GlcNAcβ1-6(3-deoxy-Galβ1-3) GalNAcα-Bn.

(2) Large scale enzyme product B using 4-deoxy-Galβ1-3GalNAcα-Bn substrate eluted on Bio-Gel P-4 as well as on reversed phase HPLC separated well from the substrate. Product B did not change its elution pattern on HPLC after β-galactosidase digestion, suggesting that B is resistant to β-galactosidase. The NMR spectrum of product B (Table 4) compared with the substrate indicate the addition of GlcNAcβ6 with a resonance at 1.962 ppm due to the *N*-acetyl group and a doublet due to H-1 at 4.542 ppm with a coupling constant $J_{1,2}$ of 8.4 Hz. The spectrum of product B showed a typical upfield shift of one of the benzyl CH_2 proton signals from 4.577 to 4.504 ppm. The masses determined by FAB mass spectrometry were *m/z* 458 $[M + H]^+$ and 480 $[M + Na]^+$ for the substrate 4-deoxy-Galβ1-3GalNAcα-Bn and *m/z* 661 $[M + H]^+$ and 683 $[M + Na]^+$ for product B. Methylation analysis of substrate and product B indicated the presence of the 4-deoxy-Gal derivative in both cases (eluting at 11.3 min, *m/z* 115, 118, 162, 175) but no GalNAc derivative was detectable, possibly due to the resistance of the benzyl group to acid hydrolysis. The data are consistent with the structure [^{14}C]GlcNAcβ1-6(4-deoxy-Galβ1-3)GalNAcα-Bn for product B.

(3) Large scale enzyme product C from Galβ1-3(2-deoxy)Galα-Bn substrate was isolated similarly to products A and B. The NMR data of product C are shown in Table 4. Product C shows resonances at 2.096 ppm and 4.524 ($J_{1,2}$ = 8.4 Hz) due to the *N*-acetyl group and H-1, respectively, of a GlcNAcβ6 residue. The H-2, H-3, and H-4 signals of the Galα residue experienced an upfield shift in the product; one of the benzyl CH_2 protons also underwent an upfield shift from 4.574 to 4.519 ppm. Upon β-galactosidase digestion of product C, the elution time of radioactivity on HPLC changed from 36 min to 48 min, as expected for a smaller oligosaccharide. This shows that product C is susceptible to β-galactosidase digestion and GlcNAc is not added to the Galβ residue but rather to the Galα residue of Galβ1-3(2-deoxy)Galα-Bn. The masses determined by FAB mass spectrometry were *m/z* 417 $[M + H]^+$ for the substrate Galβ1-3(2-deoxy)Galα-Bn and *m/z* 620 $[M + H]^+$ for product C. Methylation analysis showed the permethylated alditol acetate derivatives from terminal GlcNAc (eluting at 15.8 min, *m/z* 115, 117, 143, 145, 159, 161, 202, 205), terminal galactose (eluting at 12.6 min, *m/z* 118, 129, 145, 161, 162, 205) and 3,6-substituted 2-deoxy-Gal (eluting at 14.4 min, *m/z* 118, 129, 145, 160, 189, 204). No 6-substituted hexose or 3-substituted 2-deoxyhexose were detected. These data are consistent with the structure [^{14}C]GlcNAcβ1-6(Galβ1-3)(2-deoxy)-Galα-Bn for product C.

Enzyme products using 3-deoxy-Galβ1-3GalNAcα-Bn, 4-deoxy-Galβ1-3GalNAcα-Bn and Galβ1-3(2-deoxy)Galα-Bn substrates made by core 2 β6-GlcNAc-T in the remaining tissues eluted at identical times to products A, B and C, respectively.

Inhibition of core 2 β6-GlcNAc-transferase

None of the analogues was capable of appreciable inhibition of core 2 β6-GlcNAc-T when mixed at equimolar concentrations with the Galβ1-3GalNAcα-Bn substrate using CML cells, AML cells, pig gastric mucosa, rat and human colon, mouse kidney or hen oviduct as the enzyme source. The amount of enzyme product formed in the presence of analogues which were themselves substrates was that expected for two competing substrates. The inactive analogues 6-deoxy-Galβ1-3GalNAcα-Bn, Galβ1-3(4-deoxy)-GalNAcα-Bn, Galβ1-3(2-deoxy)Galα-Bn and Galβ1-3[6-(4,4-azo)pentyl]GalNAcα-Bn in tenfold excess over the substrate Galβ1-3GalNAcα-Bn also did not inhibit the enzyme from CML and AML cells significantly. At high concentrations, Galβ1-3(6-deoxy)GalNAcα-Bn was a competitive inhibitor in AML cells (K_M 0.95 mM, K_I 29 mM), CML cells (K_M 2.5 mM, K_I 37 mM), human colon (K_M 1.5 mM, K_I 21 mM) and mouse kidney (K_M 2.0 mM, K_I 12 mM) but no inhibition was observed in rat colon and pig gastric mucosa (K_M 1.3 and 1.4 mM respectively).

Effect of cations of core 2 β6-GlcNAc-transferase activity

The inclusion of a divalent cation in the assay with Galβ1-3GalNAcα-Bn substrate resulted in marked differences in product formation by different tissues (Table 5). Several metal ions stimulate or support core 2 β6-GlcNAc-T activity to varying degrees, depending on the tissue. Cu^{2+} reduced the activity in all tissues (Table 5).

Tissue distribution of core 2 β6-GlcNAc-T, core 4 β6-GlcNAc-T and blood group I β6-GlcNAc-T

The β6-GlcNAc-T activities that synthesize core 2, core 4, and blood group I were assayed in various tissues (Table 6).

Table 4. 500 MHz proton NMR data with chemical shifts in ppm and coupling constants in Hz in parentheses.

	Gβ3GAαBn 3-deoxy	Gn β6 Gβ3GAαBn 3-deoxy (product A)	Gβ3GAαBn 4-deoxy	Gn β6 Gβ3GAαBn 4-deoxy (product B)	6-azopentyl GAα-Bn	6-azopentyl Gβ3GAα-Bn	Gβ3GαBn 2-deoxy	Gn β6 Gβ3GαBn 2-deoxy (product C)	Gβ3GAαBn[c]	Gn β6 Gβ3GAαBn[a]
GaINAcα										
H-1	4.974	4.976	4.970	4.972	4.97	4.971	–	–	4.97	4.97
H-2	4.312 (3.7, 11.3)	4.317	4.307 (3.8, 11.2)	4.312 (3.8, 11.1)	4.12	4.309	–	–	4.31	4.31
H-3	4.07	4.0	4.038	4.01	3.92	4.033	–	–	4.04	4.02
H-4	4.251	4.221	4.230	4.204	3.96	4.208	–	–	4.25	4.23
H-5	4.06	4.1	4.046	nd	4.12[b]	4.15[b]	–	–	4.06	4.15
H-6	3.7	nd	3.5–3.7	nd	3.65	3.5–3.7	–	–	3.74	3.70
H-6′	3.7	nd	3.5–3.7	nd	3.65	3.5–3.7	–	–	3.74	4.07
R-acetyl	1.955	1.970[c]	1.964	1.965[c]	1.968	1.967	–	–	1.96	1.96
Galβ										
H-1	–	–	–	–	–	4.454 (7.8)	4.486 (7.8)	4.463 (7.9)	4.45	4.43
H-2	–	–	–	–	–	nd	3.519 (8.3)	3.54	3.51	3.50
H-3	–	–	–	–	–	nd	3.634 (3.4, 9.9)	nd	3.61	3.60
H-4	–	–	–	–	–	3.904	3.910 (3.4)	3.906	3.90	3.89
3/4-deoxyGalβ										
H-1	4.466 (7.9)	4.443 (7.9)	4.442 (7.8)	4.42 (7.8)	–	–	–	–	–	–
H-2	3.69	nd	3.183	3.173	–	–	–	–	–	–
H-3 ax., eq.	1.694, 2.16	1.69, nd	3.7	nd	–	–	–	–	–	–
H-4	3.97	nd	1.418, 1.9	1.4, nd	–	–	–	–	–	–
2-deoxy-Galα										
H-1	–	–	–	–	–	–	5.204	5.200	–	–
H-2 ax., eq.	–	–	–	–	–	–	2.02	2.01	–	–
H-3	–	–	–	–	–	–	4.196 (2.8, 8.7)	4.169	–	–
H-4	–	–	–	–	–	–	4.116	4.079	–	–
GlcNAcβ										
H-1	–	4.544 (8.7)	–	4.542 (8.4)	–	–	–	4.524 (8.4)	–	4.54
H-2	–	nd	–	nd	–	–	–	3.73	–	3.74
R-acetyl	–	1.964[c]	–	1.962	–	–	–	2.096	–	1.97
CH_2 Bn	4.574 (11.6) 4.76	4.505 (11.3) 4.75	4.577 (11.9) 4.689 (11.7)	4.504 (11.4) 4.68	4.6 4.7	4.587 (11.9) 4.686	4.574 (11.5) 4.72	4.519 (11.1) 4.66	4.57 4.77	4.50 4.71

[a] Data from A. Pollex-Krüger *et al.* [49]; nd, not determined.
[b] Tentative data.
[c] Signals may be interchanged.

Table 5. Effect of divalent cations on core 2 β6-GlcNAc-transferase activity from AML cells and from mucin secreting tissues.[a]

Addition of metal ion (10 mM)	*Enzyme activities* (%)				
	AML	*PG*	*HC*	*RC*	*MK*
EDTA	100	100	100	100	100
Fe^{2+}	120	203	174	163	137
Ca^{2+}	91	156	134	149	126
Mn^{2+}	89	85	81	138	117
Mg^{2+}	78	127	93	177	96
Zn^{2+}	50	34	75	114	91
Co^{2+}	38	35	67	118	77
Cu^{2+}	4	5	21	9	59

[a] Assays were carried out as described in the Materials and methods section by HPLC (as described in Table 3). AML, PG, HC, RC, MK as described in Table 2.

Table 6. *O*-Glycan core 2, core 4 and blood group I β6-GlcNAc-transferase activities in various tissues.[a]

Tissue	β6-GlcNAc-T *activities* (nmol h^{-1} mg^{-1})		
	core 2	*core 4*	*I*
AML[b]	5.2	<0.1	<0.1
CML[b]	1.2	<0.1	<0.1
human granulocytes[b]	0.3	<0.1	<0.1
CaCo-2 cells[c]	9	0.5	<0.1
MK	135	2.0	3.4
RC	96	92	41
PG	95	46	12
HC	44	2.3	10
HO	31	18	2.3

[a] Assays were carried out as described in the Materials and methods section. The substrate for core 2 β6-GlcNAc-transferase was 2 mM Galβ3GalNAcα-Bn, for core 4 β6-GlcNAc-transferase was 2 mM GlcNAcβ3GalNAcα-Bn, and for blood group I β6-GlcNAc-transferase was 2 mM GlcNAcβ3Galβ-methyl. MK, RC, PG, HC, HO, as described in Table 2.
[b] Values are from Brockhausen *et al.* [8].
[c] Values are from Brockhausen *et al.* [20].

The core 4 and I activities are very low or undetectable in CML and AML cells, normal human granulocytes, and in CaCo-2 human colonic adenocarcinoma cells, whereas all mucin secreting tissues (rat and human colon, pig stomach and hen oviduct) and mouse kidney contained these activities at various levels.

Specificity and inhibition of α3-SA-T

The substrate specificity of the human placental α3-SA-T was measured with Galβ1-3GalNAc- and related substrates with hydrophobic and peptide aglycon groups as well as with the deoxy analogues of Galβ1-3GalNAcα-Bn (Tables 7 and 8). Substrates activity was in the range of 0.6–3.8 nmol h^{-1} mg^{-1} (Table 7). An aglycon group does not appear to be required since Galβ1-3GalNAc was a good substrate. Galβ1-3GlcNAcβ-Bn was a poor substrate for the placental α3-SA-T. Compounds with core 2 structure GlcNAcβ1-6(Galβ1-3)GalNAcα- and substitution or deletion of the 6-hydroxyl of galactose were active substrates (Table 7). Deoxy derivatives showed considerable activity but relatively high K_M values with the exception of 3-deoxy-Galβ1-3GalNAcα-Bn, which was not active (Table 8). Galβ1-3GalNAcα-Bn was found to have a similar V_{max} but a much lower K_M value (0.03 mM) than any of the deoxy derivatives (Table 8). The V_{max}/K_M of this standard substrate is therefore much higher (57) than the value for the deoxy derivates (<5).

Table 7. Specificity of purified human placental α3-sialyltransferase using Galβ1-3GalNAcα-R substrate and substrate analogues.

Substrate or analogue (2 mM)	*Activity* (nmol h^{-1} μl^{-1}[a])
Galβ1-3GalNAcα-Bn	1.7
Galβ1-3GalNAcα-pnp	1.6
Galβ1-3GalNAcα-onp	0.9
Galβ1-3GalNAcα-phenyl	2.1
Galβ1-3GalNAcα-mco	0.7
Galβ1-3GalNAcα-Ser	0.7
Ala-Pro-(Galβ1-3GalNAcα-)Ser-Ser-Ser	0.9
Ala-Pro-(Galβ1-3GalNAcα)Thr-Ser-Ser	1.0
Galβ1-3GalNAcα-AFGP	1.0
Galβ1-3GlcNAcβ-Bn	0.1
GlcNAcβ1-6(Galβ1-3)GalNAcα-Bn	0.6
GlcNAcβ1-6(GlcNAcβ1-6Galβ1-3)GalNAcα-Bn	0.7
GlcNAcβ1-6(D-Fucβ1-3)GalNAcα-Bn	1.4
3-deoxy-Galβ1-3GalNAcα-Bn	<0.1
4-deoxy-Galβ1-3GalNAcα-Bn	0.6
6-deoxy-Galβ1-3GalNAcα-Bn	1.3
Galβ1-3(4-deoxy)GalNAcα-Bn	1.3
Galβ1-3(6-deoxy)GalNAcα-Bn	1.6
Galβ1-3(2-deoxy)Galα-Bn	0.2
Galβ1-3GalNAc-[b]	3.8
Galβ1-3GalNAcα-Bn[b]	2.6

[a] Sialyltransferase assays were carried out as described in the Materials and methods section by high voltage electrophoresis.
[b] Results from a separate experiment.

In contrast, the α3-SA-T from AML cells was found to have a K_M value for the Galβ1-3GalNAcα-Bn substrate similar to those for the deoxy derivatives (Table 8). The 3-deoxy-Galβ1-3GalNAcα-Bn analogue was not a substrate. Galβ1-3[6-(4,4-azo)pentyl]GalNAcα-Bn was a relatively good substrate with a K_M of 0.5 mM and a V_{max} of 12.5 nmol h^{-1} mg^{-1} (Table 8) and did not inhibit α3-SA-T activity. Both the placental and the AML α3-SA-T were inhibited by 3-deoxy-Galβ1-3GalNAcα-Bn (Table 8); kinetic analysis showed competitive inhibition with K_I values of 0.8 mM and 3.1 mM, respectively. A weak inhibition was observed when 3-deoxy-Galβ1-3GalNAcα-Bn was

Table 8. Inhibition kinetics of α3-sialyltransferase from human placenta and from AML cells.

	K_M (mM)	V_{max} (nmol h^{-1} μl^{-1})	K_I (mM)	V_{max} (K_M)
Human placental α3-SA-T:				
Galβ3GalNAcα-Bn	0.03	1.7	–	57
3-deoxy-Galβ3GalNAcα-Bn	–	–	0.8	–
4-deoxy-Galβ3GalNAcα-Bn	0.5	0.6	–	1.2
6-deoxy-Galβ3GalNAcα-Bn	3.4	2	–	0.6
Galβ3(4-deoxy)GalNAcα-Bn	0.4	1.7	–	4.3
Galβ3(6-deoxy)GalNAcα-Bn	1.6	1.3	–	0.8
Galβ3(2-deoxy)Galα-Bn	2.7	1.7	–	0.6
AML α3-SA-T:		nmol h^{-1} mg^{-1}		
Galβ3GalNAcα-Bn	0.4	6	–	15
3-deoxy-Galβ3GalNAcα-Bn	–	–	3.1	–
4-deoxy-Galβ3GalNAcα-Bn	0.4	17	–	43
6-deoxy-Galβ3GalNAcα-Bn	0.4	18	–	45
Galβ3(4-deoxy)GalNAcα-Bn	1.3	16	–	12
Galβ3(6-deoxy)GalNAcα-Bn	1.3	10	–	7.7
Galβ3(2-deoxy)Galα-Bn	1.3	2.7	–	2.1
Galβ3[6-*O*-(4,4-azo)pentyl]GalNAcα-Bn	0.5	12.5	–	25

[a] Enzyme assays were carried out as described in the Materials and methods section by high voltage electrophoresis.

Table 9. Inhibition of α3-sialyltransferase from AML and CML cells.[a]

	Galβ3GalNAcα-Bn (mM)	3-deoxy-Galβ3GalNAcα-Bn (mM)	*Activity* (nmol h^{-1} mg^{-1})
AML patient 1	0.25	–	8.9
	0.25	2	6.4
AML patient 2	0.25	–	10.8
	0.25	2	8.1
AML patient 3	0.25	–	15.6
	0.25	2	10.7
CML patient 1	0.25	–	7.9
	0.25	2	6.9
CML patient 2	0.25	–	2.5
	0.25	2	1.6
CML patient 3	0.25	–	4.4
	0.25	2	4.4

[a] Enzyme assays were carried out as described in the Materials and methods section by high voltage electrophoresis. 3-Deoxy-Galβ1-3GalNAcα-Bn was not a substrate.

present in eightfold excess over Galβ1-3GalNAcα-Bn substrate in incubations with cells from three AML patients and from two out of three CML patients (Table 9).

Discussion

The *O*-glycan structures that a cell is capable of synthesizing are determined mainly by the specificities of glycosyltransferases. Knowledge of the specific requirements of glycosyltransferases for their substrates may allow the development of inhibitors. Inhibitors may be capable of modifying cellular *O*-glycan structures and are therefore potential therapeutic agents.

We have investigated the substrate specificities of core 2 β6-GlcNAc-T and α3-SA-T, two regulatory enzymes which are involved in the processing of *O*-glycan core 1, Galβ1-3GalNAc-, and are increased in leukemia [8, 10]. We have synthesized analogues of Galβ1-3GalNAcα-Bn to explore the enzyme binding requirements for hydroxyl groups in the substrate; these compounds have also been tested as enzyme inhibitors. Both the galactose and the GalNAc moieties of the Galβ1-3GalNAcα-Bn substrate have been

modified to yield mono-deoxy derivatives. Although both enzymes probably act on similar core/substrates *in vivo*, they exhibit different substrate specificities *in vitro*.

In five tissues assayed, 6-deoxyGalβ1-3GalNAcα-Bn, Galβ1-3(4-deoxy)GalNAcα-Bn and Galβ1-3(6-deoxy)-GalNAcα-Bn proved inactive as substrates for the core 2 β6-GlcNAc-T. Williams *et al.* [13] also reported inactivity of the canine submaxillary gland enzyme towards 6-deoxyGalβ1-3GalNAcα-Bn. These results suggest that the core 2 β6-GlcNAc-T from all tissues tested has an absolute requirement for the 4- and 6-hydroxyls of GalNAc, and the 6-hydroxyl of galactose in the substrate. Thus the enzyme cannot act after the substitution of the 6-hydroxyl of galactose. Recognition of the 3- and 4-hydroxyls of galactose and the acetamido group of GalNAc, however, is variable between enzymes from different tissues. It is interesting that the core 2 β6-GlcNAc-T from mucin-secreting tissues (named the M type enzyme), which is probably capable of acting on Galβ1-3GalNAcα-R, GlcNAcβ1-3GalNAcα-R and GlcNAcβ1-3Galβ-R to make cores 2 and 4 and the I epitope, respectively [16, 18], does not have an absolute requirement for an equatorial 4-hydroxyl on the terminal sugar residue. In contrast, the enzyme in normal and leukemic leukocytes (named the L type enzyme) has an absolute requirement for the 4-hydroxyl of galactose, consistent with its inability to act on either GlcNAcβ1-3GalNAcα-R or GlcNAcβ1-3Galβ-R. Core 2 β6-GlcNAc-T (M) from all tissues except hen oviduct is not fully active when there is neither an *N*-acetyl nor a hydroxyl group at the carbon-2 position of GalNAc. The effect of removal of the 2-hydroxyl of galactose remains to be shown. The core 2 β6-GlcNAc-T specificities in AML cells and rat colon are illustrated in Fig. 2.

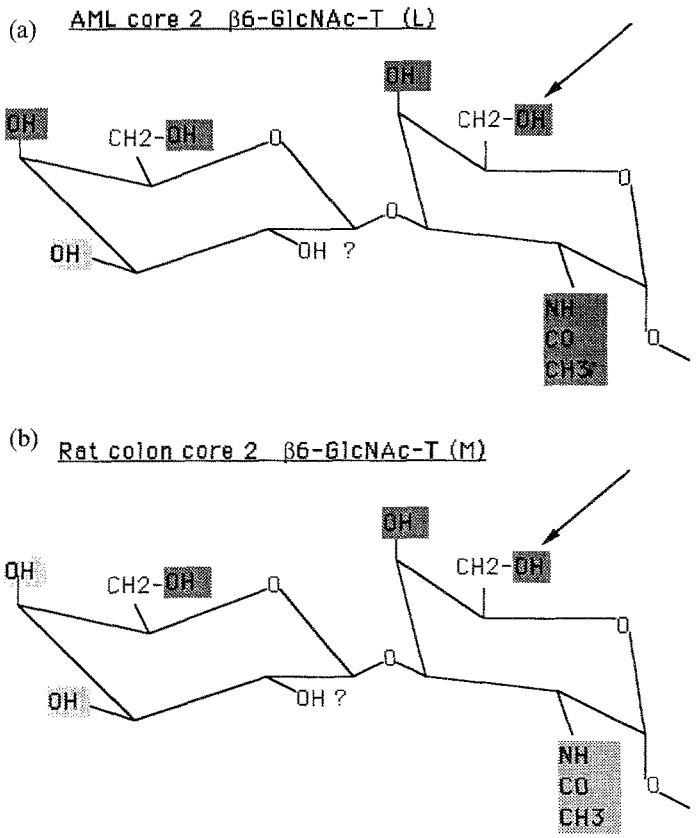

Figure 2. Structural requirements for core 2 β6-GlcNAc-transferase activity from (a) AML cells and (b) mucin secreting tissue rat colon. The shaded areas indicate structural requirements for enzyme activity. The degree of shading corresponds to the degree of requirement.

Core 2 β6-GlcNAc-T activity in pig gastric mucosa has been reported to be accompanied by two other branching activities: core 4 β6-GlcNAc-transferase converting core 3 to core 4 and blood group I β6-GlcNAc-transferase converting blood group i to I. These two activities are absent from leukemic cells and from CaCo-2 human colonic adenocarcinoma cells [20], but they are present in a purified preparation of bovine tracheal core 2 β6-GlcNAc-transferase [18] and in a number of mucin secreting tissues (Table 6) [15, 16]. The substrate specificities of core 2 β6-GlcNAc-transferases suggest that there are at least two families of similar enzymes, one restricted to core 2 synthesis (L enzyme), and one responsible for core 2, 4 and blood group I synthesis (M enzyme). The L enzyme is regulated during differentiation and activation of leukocytes. Our substrate specificity studies indicate that the activities of these enzymes appear to be controlled in a tissue-specific manner. The differences in reactivity towards 3-deoxy-Galβ1-3GalNAcα-Bn seen between homogenate and microsomes from rat colon support the possibility of a tissue-dependent factor affecting specificity. Recently, cDNA was isolated encoding an additional β6-GlcNAc-T synthesizing the I antigen with a substrate specificity distinct from that of the M enzyme [50].

Structural analysis of large scale enzyme product revealed that 3-deoxy-Galβ1-3GalNAcα-Bn and 4-deoxy-Galβ1-3GalNAcα-Bn were substrates for the core 2 β6-GlcNAc-T activities from rat colon and Galβ1-3(2-deoxy)Galα-Bn was a substrate for the enzyme in hen oviduct. In each case, the enzyme added GlcNAc in β1-6 linkage to the GalNAcα or Galα- but not the Galβ residue of 3-deoxy-Galβ1-3GalNAcα-Bn, 4-deoxy-Galβ1-3GalNAcα-Bn or Galβ1-3(2-deoxy)Galα-Bn substrate, respectively.

Susceptibility of [^{14}C]GlcNAcβ1-6(Galβ1-3)2-deoxy-Galα-Bn and resistance of enzyme products [^{14}C]GlcNAcβ1-6(3-deoxy-Galβ1-3)GalNAcα-Bn and [^{14}C]GlcNAcβ1-6(4-deoxy-Galβ1-3)GalNAcα-Bn to β-galactosidase [48] shows that bovine testicular β-galactosidase requires both the 3- and the 4-hydroxyls of the nonreducing galactose for cleavage.

None of the inactive substrates proved to be inhibitors with the exception of Galβ1-3(6-deoxy)GalNAcα-Bn, the anlogue lacking the reactive 6-hydroxyl of GalNAc, which weakly inhibited the core 2 β6-GlcNAc-T from CML and

AML cells, mouse kidney and human colon, but not from pig stomach and rat colon, in a competitive fashion. Hindsgaul *et al.* [51] also reported competitive inhibition of the mouse kidney enzyme by this compound, and suggested that this 6-hydroxyl in the substrate may form a critical hydrogen bond with the enzyme. Three more enzymes showed a similar type of competitive inhibition by substrate analogues lacking the reactive hydroxyl: pig submaxillary α2-Fuc-T, mung bean α4-Fuc-T and hamster kidney GlcNAc-transferase V, whereas four other transferases were not affected by the corresponding substrate analogues [50]. The deoxy analogues are relatively ineffective as inhibitors and have a larger K_I than K_M value. The diazirine Galβ1-3[6-*O*-(4,4-azo)pentyl]GalNAcα-Bn did not react irreversibly with the binding site of the enzyme, probably because the 6-*O*-pentyl group is sterically hindering binding.

Although the core 2 β6-GlcNAc-T does not require metal ions for activity, the addition of metal ions has considerable effects. However, the nature of these effects is not known. There is an obvious variability between tissues in the effect of metal ions on activity, but this effect does not parallel the differences in substrate specificity. It is possible that in pig stomach and in human and rat colon elongation β3-GlcNAc-transferase (path a, Fig. 1) acting on the same substrate may be activated by Mn^{2+} and Co^{2+} but not by Ca^{2+}, Mg^{2+}, Zn^{2+} and EDTA. The effect of Fe^{2+} and Cu^{2+} on the latter enzyme or its presence in mouse kidney has not yet been investigated.

The leukemic core 2 β6-GlcNAc-T (L) belongs to the class of enzymes with more restricted specificity as it is unable to make the β6-branch of core 4 and the I antigen. In rat tissues, the β6-GlcNAc-T synthesizing blood group I is accompanied by another enzyme with a different specificity in rat tissues [52]. Recently, Sekine *et al.* [53] reported a similar branching activity in mouse kidney which forms the GlcNAcβ1-6(Galβ1-3)GalNAcβ-R branch in globosides; based on the results of our specificity studies, this activity may be due to the core 2 β6-GlcNAc-T. β6-GlcNAc-T with unusual specificities acting on substrates with Galβ- terminal have been described in mouse T-cell lymphoma [54], Novikoff ascites tumour cells [55] and human serum [56]. It is possible that several different genes code for core 2 β6-GlcNAc-T activities and that these genes are under tissue-specific control.

Sialylation of core 1 (path c or d, Fig. 1) turns off the branching reaction catalysed by core 2 β6-GlcNAc-T. The reverse, however, is possible (paths b and f, Fig. 1); core 2 may be formed first and is then easily sialylated; i.e., core 2 β6-GlcNAc-T has to act before α3-SA-T. Hypersialylation of leukemic cells by α3-SA-T may thus preclude core 2 formation in the presence of high core 2 β6-GlcNAc-T activity, assuming that the intracellular localization of these transferases allows α3-SA-T to act before or at the same time as core 2 β6-GlcNAc-T. This would explain the finding

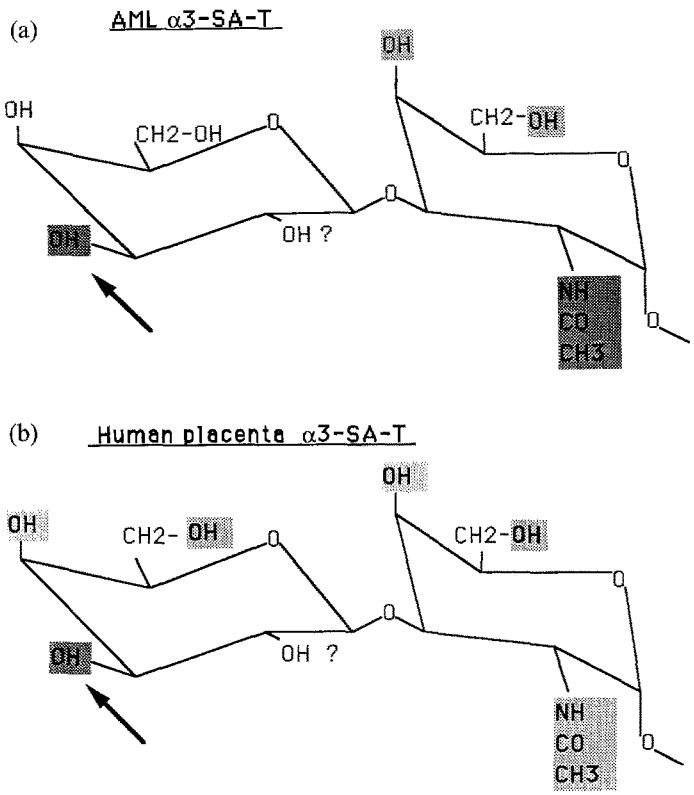

Figure 3. Structural requirements for α3-SA-transferase activity from (a) AML cells and (b) human placenta. The shaded areas indicate structural requirements for enzyme activity. The degree of shading corresponds to the degree of requirement.

of high amounts of short sialylated core 1 chains in AML cells [2] in the presence of a hyperactive core 2 β6-GlcNAc-T.

Specificity studies of the α3-SA-T from human placenta and AML cells indicate that the enzyme has an absolute requirement only for the 3-hydroxyl of the galactose residue of the substrate and can act when the other substituents of the sugar rings are removed. It can also act when the 6-hydroxyl of the galactose residue of the core 2 substrate is removed or substituted by GlcNAc. The degree of activity versus the various deoxy substrate analogues differs considerably between the enzymes from the two different cell types. This suggests that also the specificity of the *O*-glycan α3-SA-T may be regulated by tissue-specific factors. The absence of the 4-hydroxyl of galactose or GalNAc does not abolish α3-SA-T activity. The human placenta and pig submaxillary gland [27] enzymes, however, do not act on Gal1-3GlcNAc. It has not yet been established if the enzyme binds the 2-hydroxyl of the galactose residue of core 1 and 2 substrates. The substrate binding requirements of α3-SA-T from AML cells and placenta are illustrated in Fig. 3.

Both the core 2 β6-GlcNAc-T and the α3-SA-T are competitively inhibited by the corresponding substrate analogues lacking the hydroxyl that is acted upon. Hindsgaul

et al. [50] reported lack of inhibition by the 3-deoxy analogue of the substrate for the α3-SA-T acting on N-glycans; this enzyme is different from the *O*-glycan α3-SA-T. Since core 2 β6-GlcNAc-T and *O*-glycan α3-SA-T may be distinguished by their requirements for substituents of the substrate, compounds may be devised that selectively bind to either one of the two enzymes. This strategy was used to design Galβ1-3[6-*O*-(4,4-azo)pentyl]GalNAc-Bn as a selective inhibitor. However, the derivative did not bind or react with the core 2 β6-GlcNAc-T. In addition, it bound but the diazirine group did not react with nucleophilic groups in the α3-SA-T, since it was a substrate but not an inhibitor for the α3-SA-T. In future syntheses of potential inhibitors, we will attach reactive groups at other positions in the sugar rings, as well as the aglycon portion of the substrate, to create compounds that distinguish between the core 2 β6-GlcNAc-T and the α3-SA-T and are more potent than the present inhibitors.

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